

Identification of DNA cleavage- and recombination-specific hnRNP cofactors for activation-induced cytidine deaminase

Wenjun Hu¹, Nasim A. Begum¹, Samiran Mondal, Andre Stanlie, and Tasuku Honjo²

Department of Immunology and Genomic Medicine, Graduate School of Medicine, Kyoto University, Yoshida Sakyo-ku, Kyoto 606-8501, Japan

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Activation-induced cytidine deaminase (AID) is essential for antibody class switch recombination (CSR) and somatic hypermutation (SHM). AID originally was postulated to function as an RNA-editing enzyme, based on its strong homology with apolipoprotein B mRNA-editing enzyme, catalytic polypeptide 1 (APOBEC1), the enzyme that edits apolipoprotein B-100 mRNA in the presence of the APOBEC cofactor APOBEC1 complementation factor/APOBEC complementation factor (A1CF/ACF). Because A1CF is structurally similar to heterogeneous nuclear ribonucleoproteins (hnRNPs), we investigated the involvement of several well-known hnRNPs in AID function by using siRNA knockdown and clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9-mediated disruption. We found that hnRNP K deficiency inhibited DNA cleavage and thereby induced both CSR and SHM, whereas hnRNP L deficiency inhibited only CSR and somewhat enhanced SHM. Interestingly, both hnRNPs exhibited RNA-dependent interactions with AID, and mutant forms of these proteins containing deletions in the RNA-recognition motif failed to rescue CSR. Thus, our study suggests that hnRNP K and hnRNP L may serve as A1CF-like cofactors in AID-mediated CSR and SHM.

class switch recombination | somatic hypermutation |
activation-induced cytidine deaminase | B cell | IgH

Antigen-stimulated mature B cells express activation-induced cytidine deaminase (AID), an essential enzyme for somatic hypermutation (SHM) and class switch recombination (CSR) at the Ig locus (1, 2). AID induces DNA breaks at the variable (V) and switch (S) regions during SHM and CSR, respectively. Most of the mutations produced during SHM are introduced by error-prone DNA synthesis during single-strand break (SSB) repair (3, 4). In contrast, CSR requires the conversion of SSBs to double-strand breaks (DSBs), followed by recombination between two DSB ends located in the donor and acceptor S regions (5, 6). The entire process of CSR is accomplished through elaborate DNA-repair processes involving S-S synapse formation and end-joining. The mechanism by which AID functions differently in DNA cleavage and recombination at different loci remains unclear. Functional studies of a large number of AID mutants revealed that the N-terminal AID mutations impair SHM and CSR, indicating that the AID N terminus, which also possesses a bipartite nuclear-localization signal, is required for DNA cleavage in both SHM and CSR (7–9). On the other hand, C-terminal AID mutations suppressed the recombination activity of CSR but had no effect on SHM, indicating that the C terminus of AID, which contains a nuclear-export signal, is required for the recombination activity associated specifically with CSR (7, 9, 10). Indeed, recent studies showed that defects in the AID C terminus compromise DNA end-joining and S-S synapse formation without perturbing DNA breakage at either the V or S region (11, 12), also suggesting a specific role for the AID C terminus in the recombination step of CSR. Given AID's small size (198 residues), we were intrigued by its diverse and compartmentalized functions.

AID is a member of the AID–apolipoprotein B mRNA-editing enzyme, catalytic polypeptide (APOBEC) cytidine deaminase

family, which is related to ancestral AID-like enzymes, PmCDA1 and PmCDA2, expressed in the lamprey (13, 14). Although most of these related proteins are predicted to be involved in cytidine deamination, their targets and the molecular mechanisms are not fully elucidated (15). The best-characterized AID-like enzyme is APOBEC1, an RNA-editing enzyme that catalyzes the site-specific deamination of C to U at position 6666 of the apolipoprotein B-100 (APO B-100) mRNA, generating a premature stop codon (16–18). The edited mRNA, referred to as “APOB-48,” encodes the triglyceride carrier protein, a truncated product of the LDL carrier protein, which is encoded by APO B-100 mRNA.

The specificity of the APOBEC1 RNA-editing activity is determined by its cofactor, APOBEC1 complementation factor/APOBEC complementation factor (A1CF/ACF), a 64-kDa RNA-binding protein containing three distinct RNA-recognition motifs (RRMs) (19–23), which specifically targets a single C within a transcript of ~14,000 bases. A conserved motif of 11 nucleotides (referred to as the “mooring sequence”), located four to six nucleotides downstream of the edited base, is critical for A1CF binding and RNA editing (24, 25). It also has been suggested that a 30-bp predicted stem-loop structure surrounding the deaminated C is involved in target-site selection, but this suggestion is controversial (26, 27). A1CF has been hypothesized to melt the secondary structure of the editing site (28), thus allowing C6666 access to APOBEC1 deamination and that A1CF is the critical factor in the APOBEC1-editing complex (21, 22, 29) that mediates the RNA-substrate recognition and base-editing specificity. Because A1CF binds to RNA and also docks

Significance

The B-cell-specific antibody gene-diversifying enzyme activation-induced cytidine deaminase (AID) shows high homology with apolipoprotein B mRNA-editing enzyme catalytic polypeptide 1 (APOBEC1), which edits apolipoprotein B-100 mRNA in the presence of cofactor APOBEC1 complementation factor/APOBEC complementation factor (A1CF/ACF). Here we show that the DNA cleavage and recombination functions of AID depend critically on its RNA-dependent interaction with distinct heterogeneous nuclear ribonucleoprotein (hnRNP) cofactors. Depletion of hnRNP K inhibited DNA cleavage essential to both class switch recombination (CSR) and somatic hypermutation, but depletion of hnRNP L blocked the CSR-associated recombination. Thus this is the first report, to our knowledge, identifying A1CF-like hnRNP-family editing cofactors for AID.

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¹W.H. and N.A.B. contributed equally to this work.

²To whom correspondence should be addressed. Email: honjo@mfour.med.kyoto-u.ac.jp.

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Although the interaction between $E\mu$ and $E\alpha$ was slightly enhanced by CIT stimulation, it was not reduced by the depletion of either hnRNP K or hnRNP L. However, the association between $S\mu$ and $S\alpha$ was reduced dramatically when hnRNP K, but not hnRNP L, was depleted. The same phenomenon was observed when the association between $E\mu$ and $S\alpha$ was examined, confirming that synapse formation at both $S\mu$ – $S\alpha$ and $E\mu$ – $S\alpha$ was disrupted by depletion of hnRNP K but not hnRNP L (Fig. 3D). Notably, the efficiency of synapse formation was elevated by hnRNP L depletion, which also correlated with the elevated γ H2AX signal in the absence of hnRNP L (Fig. 3A). It is possible that hnRNP L is involved in the end-joining step after synapse formation between the cleaved ends. If so, the lack of end repair may prolong synapsis and the processing of DSBs in the absence of hnRNP L. Thus, four independent lines of evidence support the conclusion that AID-induced DNA cleavage requires hnRNP K but not hnRNP L.

KH Domains of hnRNP K Are Required for Its Function in CSR. The KH domains of hnRNP K are known to be responsible for its RNA-binding activity (30, 40). To evaluate the importance of these domains in CSR, we constructed KH3-domain-deleted hnRNP K mutants (Fig. 4A). The mutant and WT constructs were modified so that they were resistant to the hnRNP K-targeted siRNA and were tagged at the C terminus with the c-Myc-FLAG epitope. We next examined their CSR complementation efficiencies in K2-20 cells treated with hnRNP K siRNA. The introduction of WT hnRNP K (wt- K^R , where “R” denotes resistance to siRNA-mediated degradation) successfully rescued the effect of hnRNP K depletion on IgA switching (Fig. 4B and C), whereas the KH3-domain-deleted mutant showed less CSR rescue than WT hnRNP K. We confirmed the protein expression level of each of the constructs when the endogenous hnRNP K was depleted (Fig. 4D and Fig. S5D).

The CSR rescue abilities of the other hnRNP K KH-domain mutants also were examined in CH12F3-2A cells after siRNA-mediated knockdown of endogenous hnRNP K (Fig. S5). The results showed that none of the KH-domain-deleted mutants could rescue CSR completely, indicating that the full CSR activity of hnRNP K requires the presence of all the KH domains. However, the presence of a single KH domain was sufficient for partial CSR rescue. To verify further the importance of KH domains in AID-induced DNA break, we examined the ability of wt- K^R and the mutant with KH3-domain deletion (Δ KH3 K^R) to complement DNA breaks (Fig. 4E). As expected, cotransfection of wt- K^R significantly counteracted the sihnRNP K-mediated reduction of the S region DNA break and elevated the DNA-break signal close to the WT level. In contrast, introduction of mutant Δ KH3 K^R barely counteracted the inhibition of DNA breaking caused by hnRNP K depletion. Taking these findings together, we conclude that KH-type RRM domains of hnRNP K are critical to AID’s function in DNA breaking, an indispensable and early step for both SHM and CSR.

Atypical RRM Domains of hnRNP L Are Required for Its Function in CSR. Next, we used a similar approach to determine the structural requirements of hnRNP L for CSR. As shown above, the introduction of hnRNP L siRNA into L11 cells strongly inhibits CSR. The introduction of a construct expressing siRNA-resistant c-myc-FLAG-tagged WT hnRNP L (wt- L^R) into the hnRNP L-depleted cells complemented the CSR defect very efficiently, especially at 48 h after transfection (Fig. 5A and B). Then constructs expressing siRNA-resistant hnRNP L mutants with various RRM-domain deletions (Fig. 5C) were prepared, and their protein expression levels were confirmed (Fig. 5E). Mutants lacking any one of the RRM domains were partially defective in the CSR rescue function (Fig. 5D), and the mutant devoid of all four RRMs (Δ all- L^R) lost all CSR-rescue ability. These findings suggest that an RNA-dependent activity is involved in hnRNP L’s CSR activity and show that the presence of only a single RRM domain in hnRNP L supports partial CSR recovery.

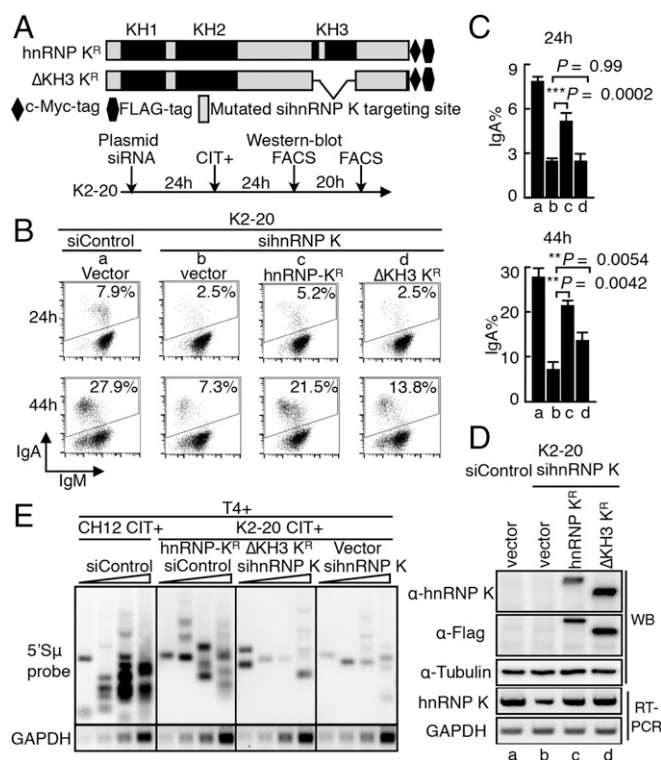


Fig. 4. Requirement for the RNA-binding domains of hnRNP K for CSR. (A) Representation of the siRNA-resistant and epitope (c-Myc-FLAG)-tagged hnRNP K constructs. WT and KH3-deleted hnRNP K are designated as hnRNP K^R and Δ KH3 K^R , respectively. (B) CSR complementation assay performed by cotransfecting K2-20 cells with K^R constructs and hnRNP K siRNA. The percentages of IgA-switched cells are indicated in the FACS profiles. (C) Efficiency of CSR rescue calculated from three independent experiments. (D, Upper) Western blot (WB) analysis shows the protein expression of the indicated K^R constructs in K2-20 cells. (Lower) RT-PCR analysis of hnRNP K mRNA expression using primers M4 and M5 (Fig. S2C). (E) Southern blot analysis of LM-PCR-based DNA break assay using T4 polymerase-treated (T4⁺) DNA samples as in Fig. 3. K2-20 cells were cotransfected with the indicated siRNAs and the hnRNP K-expressing constructs resistant to sihnRNP K-mediated degradation. After 24 h of transfection, cells were stimulated with CIT and were harvested for DNA break assay 24 h later.

RNA-Dependent Interactions of hnRNP K and hnRNP L with AID. We hypothesized that AID may form specific complex(es) with hnRNP K and hnRNP L, analogous to the APOBEC1–A1CF–RNA complex (23, 41, 42). Given that AID’s function appears to be dependent on the RNA-binding domains of hnRNP K and L, it is likely that the AID–hnRNP complex contains specific target RNA and plays an important role in RNA-substrate recognition. To examine interactions among AID, hnRNP, and RNA, we used the PAR-CLIP (photoactivatable ribonucleoside-enhanced cross-linking and immunoprecipitation) technology, which detects stable RNA–protein complexes formed by UV cross-linking of 4-thio uracil (4-SU)-labeled RNA and its associated proteins (43). To allow pull-down of the cross-linked RNP complexes with the anti-FLAG antibody, we generated constructs for expressing C-terminal Flag-tagged AID (Fig. 6A). The PAR-CLIP assay was performed using HEK293T cells expressing FLAG-tagged WT AID or mutant forms of AID containing C- or N-terminal deletions. All the constructs expressed well, and AID was immunoprecipitated quite efficiently (Fig. 6B). Both hnRNP K and L were easily detected in association with WT AID and with the C-terminally defective AID mutants (P20 and JP8Bdel). However, little binding of the hnRNPs was detected in association with the N-terminally truncated loss-of-function mutants, suggesting that the N terminus plays a crucial role in the formation of the

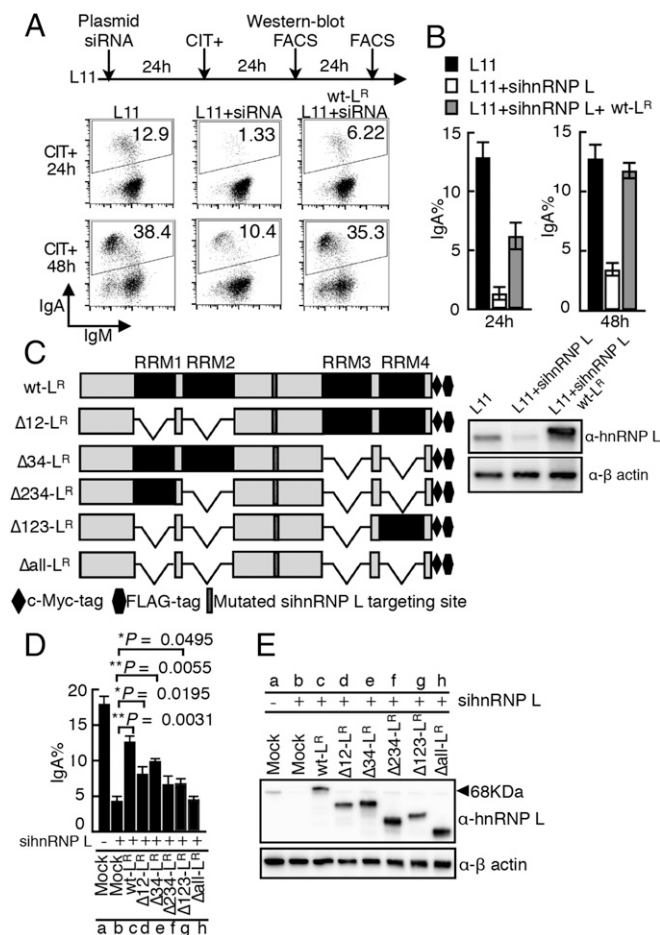


Fig. 5. The RNA-binding domains of hnRNP L are required for CSR. (A) CSR complementation assay performed by cotransfecting L11 cells with the siRNA-resistant WT hnRNP L construct (wt-L^R) and hnRNP L siRNA. The percentages of IgA switched cells are indicated in the FACS profiles. (B) CSR rescue efficiencies from three independent experiments. A representative Western blot analysis shows the degradation of endogenous but not exogenous hnRNP L (wt-L^R). (C) Representation of the various hnRNP mutants used in CSR complementation experiments. (The “Δ” with numbers indicates the specific RRM domain deleted.) (D) Summary of CSR rescue efficiencies of the hnRNP L mutants from three independent experiments. (E) Western blot analysis shows the protein expression associated with each of the L^R constructs.

AID–hnRNP complex (Fig. 6B). In addition, RNase treatment completely abolished the interactions between AID and hnRNP K or hnRNP L, showing that these interactions are completely RNA dependent (Fig. 6C). We also examined the expression of various hnRNP proteins in B cells and found that none of them were induced upon CIT stimulation (Fig. S6), suggesting that the induction of AID expression may lead to the formation of specific RNP–cofactor complexes.

Discussion

Here we investigated the role of the A1CF-like hnRNP proteins in AID-mediated CSR and SHM. We found that two hnRNPs, K and L, exhibited differential regulatory effects on the two genetic events. HnRNP K deficiency reduced both CSR and SHM, whereas the absence of hnRNP L blocked only CSR without perturbing SHM at either the V or S region. Two independent methods of estimating DNA breakage (biotin-dUTP end labeling and LM-PCR), as well as the evaluation of γ H2AX accumulation and break-end synapse formation at S regions, confirmed that hnRNP K is required for the formation of AID-dependent DNA breaks, resulting in the loss of CSR and SHM in hnRNP

K-deficient cells. In contrast, hnRNP L depletion led to enhanced, rather than reduced, γ -H2AX accumulation and to increased S–S synapse formation. Thus, hnRNP K appears to function as an AID cofactor during the formation of DNA breaks, which are required for both CSR and SHM, whereas hnRNP L is involved primarily in regulating the recombination step of CSR.

Analysis of the role of the RNA-binding motifs in hnRNP K and L indicated that all the motifs are required for full CSR activity, suggesting that CSR/SHM regulation by hnRNP cofactors involves an RNA-dependent interaction with AID. Consistent with this theory, we also found that the interaction of AID and its hnRNP cofactors is susceptible to RNase-induced dissociation. Furthermore, the N terminus of AID appears to play a critical role in the interactions, because an N-terminal truncated loss-of-function form of AID loses its ability to bind the hnRNPs.

Recently, hnRNP Q6 was identified as an APOBEC1-interacting protein that is required for APOBEC1's association with IL-8 mRNA (44). In that study, the complex was involved in IL-8 mRNA stabilization by an as yet unknown mechanism, suggesting that the APOBEC1–hnRNP Q6 complex may have a novel function associated with RNA processing rather than with C-to-U editing. More recently, mice lacking RBM47, an RNA-binding protein with three RRMs, were shown to be defective in APO B-100 editing mediated by APOBEC1 (45). These findings suggest that APOBEC1 RNA-editing complexes may comprise multiple RNA-binding or hnRNP cofactors that are involved in APO B-100 editing. Therefore, similar to APOBEC1, which forms a dimer in the APOBEC1–A1CF RNA editing complex (46), AID may form cofactor-specific RNP complexes to mediate its DNA cleavage and recombination functions. In addition, we cannot exclude the possibility that other hnRNP/RNA-binding proteins also are required for the editosome formation and AID's function.

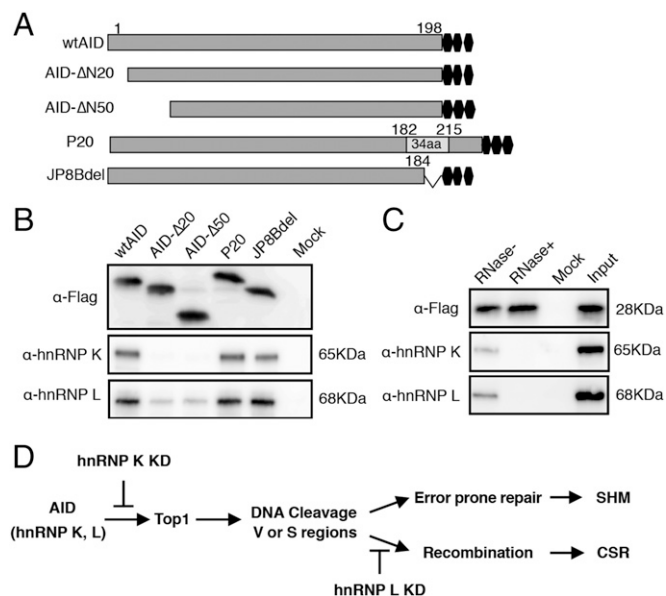


Fig. 6. RNA-dependent interactions of AID with hnRNP K and L. (A) Schematic view of the 3xFLAG-tagged WT AID and C- or N-terminal deletion mutants expressed in HEK293T cells and used in the PAR-CLIP assay. (B) Western blot analysis of the anti-Flag immunoprecipitations from 293T cells expressing WT or mutant FLAG-tagged AID and treated with 4-SU. The results show the association of the various forms of AID with hnRNP K and L and that the AID N terminus is required for their association. (C) RNase treatment of the cell lysates prevents AID association with hnRNPs. Neither hnRNP K nor hnRNP L coprecipitated with AID if the cell lysates were pretreated with RNase. (D) AID in association with an hnRNP (K or L) complex may edit specific RNAs and regulate DNA cleavage and recombination steps independently.

In conclusion, we postulate that AID may use specific hnRNPs as A1CF-like cofactors to mediate its various RNA-dependent functions. We favor the idea that the AID complex containing hnRNP K may be involved in modulating topoisomerase 1 levels, possibly through miRNA modification, resulting in the DNA cleavage associated with SHM and CSR (Fig. 6D) (47, 48). The recombination-specific editing complex comprising AID and hnRNP L may support the CSR recombination step (Fig. 6D), including the process of end-joining, through mRNA modification, followed by the formation of a new protein.

Materials and Methods

CH12F3–2A cells expressing Bcl2 and BL2 (human Burkitt's lymphoma) cells were cultured in RPMI medium 1640 (Invitrogen) supplemented with 10%

(vol/vol) FBS and penicillin-streptomycin. HEK 293T cells were cultured in DMEM (Invitrogen) supplemented with 10% (vol/vol) FBS.

Detailed materials and methods (CSR assay, siRNA oligonucleotide transfection, SHM analyses, ChIP assay, DNA break assays, 3C assay, and AID immunoprecipitation) are described in *SI Materials and Methods*. Primers and siRNAs, siRNA oligos, antibodies, and constructs are listed in *Tables S1–S4*, respectively.

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Supporting Information

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SI Materials and Methods

CRISPR/Cas9-Mediated Gene Disruption. To design the guide RNAs (gRNAs) for hnRNP gene targeting, a software tool (crispr.genome-engineering.org) predicting unique target sites throughout the mouse genome was used. Three targeting sites were selected in each of the genes encoding hnRNP K and hnRNP L. Paired oligonucleotides designed for each target site (Table S1) were annealed and cloned into the linearized GeneArt CRISPR Nuclease CD4 Reporter Vector (Invitrogen). The three targeting constructs were mixed at a 1:1 ratio, a total of 3 μ g was transfected into CH12F3-2A cells, and the CD4⁺ cells were sorted 48 h later. The CD4⁺ cells were subjected to serial dilutions and cultured for 7 d to obtain single clones. For each targeting experiment, 90 clones were selected for screening by genomic DNA analysis, protein expression, and CSR assay. The clones containing defects in the hnRNP K and hnRNP L genes were designated “K2-20” and “L11,” respectively.

hnRNP K, hnRNP L, and AID Constructs. To generate hnRNP K/hnRNP L–cMyc–FLAG fusion constructs, mouse hnRNP K (NM_025279.1) and hnRNP L (NM_177301.5) were amplified by RT-PCR and cloned into the EcoRI and XhoI sites of the pCMV6-Entry vector (Origene). Additionally, the hnRNP K–cMyc–FLAG fragment was amplified and inserted into the NheI and KpnI sites of the pcDNA3.1-Zeo vector (Invitrogen). To generate siRNA-resistant constructs, the siRNA-targeting sequences in hnRNP K/L were modified (Table S1). We also generated C-terminal 3xFLAG fusion constructs of AID and its N/C-terminal mutants; the coding sequence of human AID (NM_020661.2) was amplified by RT-PCR and cloned into the EcoRI and ClaI sites of the 3xFLAG (C-terminal)–pCMV vector (Sigma).

CSR Assay and siRNA Oligonucleotide Transfection. CH12F3-2A cells and their derivatives, the K2-20 and L11 clones, were stimulated by CIT to induce class switching as previously described (1, 2). Electroporation (Amaxa) was used to introduce siRNA oligonucleotides (Invitrogen) (Table S2) into the cells. The transfected cells were cultured for 24 h before the addition of CIT and were subjected to FACS analysis after 24 h of CIT stimulation. FITC-conjugated anti-IgM (eBioscience) and phycoerythrin (PE)-conjugated anti-IgA (Southern Biotech) antibodies were used for surface IgM and IgA staining, respectively.

To perform CSR complementation assays for hnRNP K, siRNA-resistant, epitope (cMyc–FLAG)-tagged WT or mutant hnRNP K constructs were cotransfected with the hnRNP K siRNA into K2-20 or CH12F3-2A cells. IgA switching efficiency was monitored 24 h after CIT stimulation. Similar CSR rescue experiments were conducted by cotransfecting L11 cells with siRNA-resistant hnRNP L constructs and hnRNP L siRNA.

Cell Death and Proliferation Assay. Cells were stimulated with CIT 24 h after transfection with sihnRNP K or sihnRNP L. Twenty-four hours after stimulation, cell viability was determined by propidium iodide (PI) exclusion and FACS analysis. The cell proliferation assay was performed as described previously for CH12F3-2A cells (3, 4). Carboxyfluorescein succinimidyl ester (CFSE; Invitrogen), which labels long-lived intracellular molecules with the fluorescent dye, along with the standard cell counting, was used to monitor the cell proliferation status. Twenty-four hours after siRNA transfections, cells were labeled with CFSE (5 μ M) for 15 min at 37 °C. Portions of cells were treated separately with Aphidicolin (2 μ g/mL), a well-known

inhibitor of cell-cycle progression, which served as a positive control for proliferation arrest. Cells then were stimulated with CIT, and FACS analysis and CFSE monitoring were done 48 h after stimulation.

SHM Analyses.

S region SHM. K2-20 or L11 cells were transfected with hnRNP K or L siRNA oligonucleotides 24 h before CIT stimulation for 48 h. The IgA⁺ cells were sorted, followed by purification of genomic DNA. A 676-bp region located 5' of the core S μ region was PCR-amplified using high-fidelity PrimeSTAR DNA polymerase (Takara) with the following amplification conditions: 98 °C for 5 min, 30 cycles at 98 °C for 10 s, 58 °C for 8 s, and 72 °C for 1 min (5). The PCR product was cloned into a Zero Blunt vector (Invitrogen) for sequencing, and the subsequent mutational analysis was performed using Sequencher DNA software. For each set of reactions, 96 or more clones were sequenced bidirectionally, and only the unique mutations were counted.

V region SHM. BL2-JP8BdelER cells (6, 7) were stimulated with OHT for 24 h after siRNA introduction and were incubated for an additional 48 h in the absence of OHT. The genomic DNA was purified by phenol/chloroform extraction, and PCR was performed using PrimeSTAR DNA polymerase (TaKaRa) with the following amplification conditions: 98 °C for 5 min, 30 cycles at 98 °C for 10 s, 58 °C for 8 s, and 72 °C for 1 min. The purified PCR fragments were cloned and sequenced as described above. Only the unique mutations were counted, and the mutation frequency was calculated from the number of mutations identified per total bases analyzed. The relevant PCR primers are listed in Table S1.

ChIP Assay. The ChIP assay was performed using the ChIP-IT Express Kit (Active Motif) according to the manufacturer's instructions. In brief, 5×10^6 cells were fixed in the presence of 1% formaldehyde for 5 min at room temperature. The reaction was stopped by the addition of 0.125 M glycine. A soluble chromatin fraction containing fragmented DNA of 500–2,000 bp was obtained after cell lysis and sonication. ChIP was performed by incubating the cleared lysate with 2–3 μ g of anti- γ H2AX antibody. The immunoprecipitated DNA was analyzed by real-time PCR, and the data were normalized first to the amount of input and then to the maximum value in each dataset, as described previously (2).

DNA Break Assays.

Biotin-dUTP end labeling of DNA break ends. K2-20 or L11 cells were transfected with gene-specific siRNAs, incubated for 24 h, and then stimulated with CIT. After 24 h the switching efficiency was monitored by surface IgA staining. The live cells were collected by Percoll gradient and fixed at room temperature, and then the nuclei were permeabilized and subjected to biotin-16-dUTP incorporation at the DSBs with T4 polymerase DNA polymerase (Takara). The genomic DNA was isolated by phenol/chloroform extraction and subjected to HindIII digestion overnight. The biotinylated fragments were captured with streptavidin magnetic beads and analyzed by PCR as described previously (8). The primers for amplifying S μ , S γ 1, and β 2M loci are listed in Table S1. **LM-PCR.** The cells were stimulated for CSR as described above, and the live cells were embedded in low-melt agarose plugs and processed for linker ligation as described previously (9). The samples were treated with T4 polymerase (Takara) before linker ligation, and the ligated DNA was subjected to GAPDH DNA

PCR analysis to adjust DNA input before LM-PCR. Threefold dilutions of input DNA were amplified by KOD-FX-Neo polymerase (Toyobo). The PCR products were electrophoresed on 1% agarose gels and validated by Southern blot using a 5' Sp probe (9, 10). The primers and probe sequences are shown in Table S1.

3C Assay. The 3C assay using CH12F3-2A cells was described previously (11) and was adopted from the procedure described by Wuerffel et al. (12). In brief, 7×10^6 cells were washed with PBS and subjected to 1% formaldehyde cross-linking for 5 min at room temperature. The nuclear lysate was prepared using 500 μ L of lysis buffer following the instructions in the Active Motif ChIP Kit manual. The cross-linked chromatin was digested with HindIII overnight and then ligated with T4 polymerase DNA ligase (Takara). The ligated chromatin was treated with proteinase K and reverse cross-linked, and then the DNA was purified by phenol/chloroform extraction. PCRs were performed as described previously (9). The PCR primers are listed in Table S1.

RT-PCR Analysis. Total RNA was extracted from CH12F3-2A cells expressing Bcl2 or BL2 cells using TRIzol (Gibco BRL). The cDNA was synthesized using SuperScript II and an oligo d(T) primer, followed by real-time PCR using the SYBR Green Master Mix (Applied Biosystems) and mRNA-specific primers (Table S1).

AID Immunoprecipitation. The 293T cells (1.5×10^6) were transiently transfected with 8 μ g of the 3xFLAG-tagged human AID construct and were incubated for 34 h in medium supplemented with 100 mM 4-SU. The live cells were irradiated with 365 nm UV light 14 h later (13) and then were lysed in 200 μ L of RNA-binding protein immunoprecipitation (RIP) lysis buffer (Millipore), followed by treatment with RNase A and T1 (Ambion). The FLAG-tagged proteins in 50 μ L of lysate were immunoprecipitated with 5 μ g of anti-FLAG antibody (Sigma) bound to protein G Dynabeads. The beads were washed and resuspended in RIP wash buffer (Millipore), and the protein–RNA complexes were eluted with a 0.2-M glycine solution.

Analysis of hnRNP K- and hnRNP L-Expression-Defective Clones. We used the CRISPR/Cas9 system to generate hnRNP K- and hnRNP L-null CH12F3-2A cell lines. As depicted in Fig. S2A, exons 4–6 (arrowheads) of the hnRNP K gene were targeted simultaneously. The genomic DNA, which was extracted from several targeted clones, was subjected to PCR-based screening to identify clones with the designed deletions. PCR analysis of

clone K-2, using primers G1 and G2, resulted in 1.4- and 1.2-kb products, representing the WT and disrupted alleles, respectively (Fig. S2A and B). Sequencing of the smaller product confirmed that a 168-bp sequence encompassing the initiation codon was deleted in exon 4. The K-2 clone was subjected to another round of CRISPR/Cas9-mediated gene deletion to obtain CH12F3-2A cell lines with a homozygous hnRNP K knockout. Among the 82 candidate clones obtained, a single clone, K2-20, which lost the 1.4-kb WT band, was identified. Genomic DNA sequencing of K2-20 showed a 290-bp deletion in exon 4 in the other allele (Fig. S2B, Lower).

Western blot analysis showed no detectable hnRNP K expression in clone K2-20, compared with the parental K2 clone or WT cells (Fig. S2B, Right). However, the anti-hnRNP K antibody used in this experiment was specific to the N terminus of hnRNP K and thus may not have detected N-terminally truncated products expressed from the disrupted allele. Therefore we examined hnRNP K transcripts from clone K2-20 by RT-PCR using the M1–M5 primers and detected a C-terminal-specific product (169 bp) with the M4–M5 primer pair (Fig. S2C). As expected, the M1–M3 primer pair did not yield a 240-bp RT-PCR product specific to the N terminus in the K2-20 clone, although both the 169-bp and 240-bp products were easily detectable in WT cells (Fig. S2C). Thus, we concluded that the K2-20 clone expresses an N-terminal-truncated form of hnRNP K from one allele, which resulted in a compromised level of CSR (Fig. S2D). To deplete the remaining hnRNP K transcripts, we introduced an siRNA targeted to the KH3 region (located at the C terminus) of the hnRNP K gene into K2-20 cells and thereby reduced CSR drastically.

The hnRNP L alleles were disrupted using a strategy similar to the one described above (Fig. S2E). The first round of CRISPR/Cas9-mediated gene disruption produced clone L11 that contained a 2,834-bp deletion spanning exons 2–3 in one hnRNP L allele (Fig. S2F). We therefore performed a second round of CRISPR/Cas9-mediated disruption, targeting the other hnRNP L allele in L11 cells; however, all the isolated clones expressed levels of hnRNP L protein similar to that of the parental clone. We thus introduced an siRNA targeted to hnRNP L into the L11 cells and observed almost complete depletion of the hnRNP L protein (Fig. S2F). Because treatment of K2-20 and L11 cells with hnRNP K and L siRNAs, respectively, resulted in drastic inhibition of CSR (Fig. S2D and G), this combined blocking system was used in most of the experiments. We speculated that hnRNP K and L may be essential for cell viability. Consistent with this theory, the embryonic lethality of A1CF-KO mice has been reported (14).

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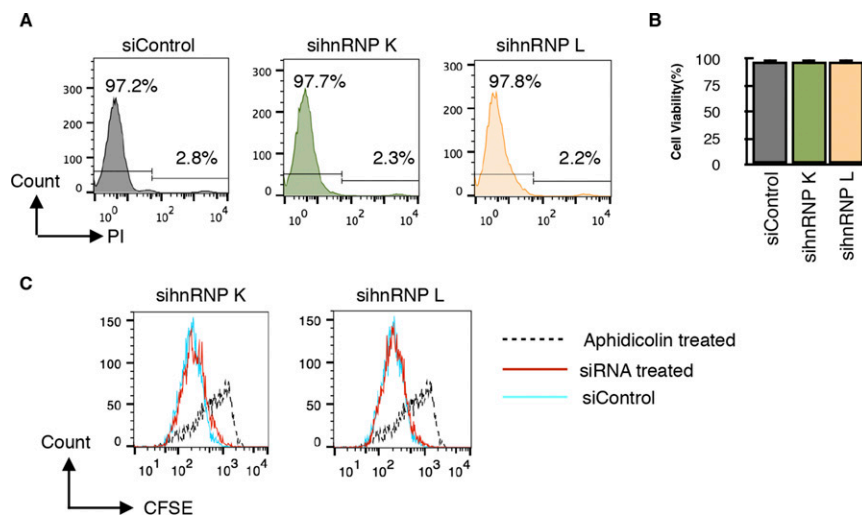


Fig. S1. Cell viability and proliferation are not affected by transient knockdown of hnRNP K or hnRNP L. (A) FACS profile of PI-stained cells 48 h after transfection. Percentages of PI⁺ and PI⁻ cells are indicated. (B) Histograms show triplicate experiments using PI⁻ cells derived from various siRNA samples as indicated. (C) Cell proliferation after siRNA transfection was measured by FACS analysis of CFSE dilution at 48 h after CIT stimulation. The profile of Aphidicolin-treated cells represents the cell proliferation arrest status (the positive control for the CFSE assay). These results are representative of three independent experiments.

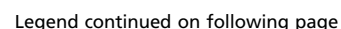


Fig. S2. Generation of hnRNP K- and hnRNP L-defective CH12F3-2A cells by CRISPR/Cas9-mediated gene disruption. (A) Schematic of Cas9/gRNA-targeting sites in the hnRNP K genomic locus. Black and white rectangles represent coding and noncoding exons, respectively. The black and white arrowheads above the exons indicate the positions of Cas9/gRNA targeting; the black arrowhead indicates the exon most effectively targeted. The arrows below the scheme show the positions of the PCR primers G1 and G2 used in *B*. (B, Upper) PCR and Western blot analyses of K2 and K2-20 clones. (Lower) Schematic representation of the deleted regions in the hnRNP K alleles in the K2-20 clone. (C, Lower) RT-PCR analysis of hnRNP K mRNA expression in WT cells and hnRNP K-defective clones. (Upper) Arrows below the scheme of the hnRNP K gene indicate the positions of PCR primers (M1–M5). (D) IgA switching efficiency of clone K2-20 after

the introduction of control and hnRNP K siRNAs. (E) Structure of the mouse hnRNP L locus showing the Cas9/gRNA-targeted regions; black arrowheads indicate the most effectively targeted exons. PCR primers G3 and G4 are shown below. (F, Upper) PCR and Western blot analyses of the hnRNP L-targeted clone, L11, with or without sihnRNP L knockdown. (Lower) Schematic representation of the deleted region in one of the hnRNP L alleles in L11. (G) IgA switching efficiency in L11 cells treated with hnRNP L siRNA.

A. Mutation analysis of 5' core S μ after CIT stimulation in hRNP K depletion cells.

Sample	Mut.clone/Total	Total Seq.	Mutation	Mut. Freq.
CH12F3-2A+siControl	35/85	57460	71	1.24E-03
K2-20+sihnRNP K	20/79	53404	35	6.55E-04

B. Mutation analysis of 5' core S_u after CIT stimulation in hRNP L depletion cells.

Sample	Mut.clone/Total	Total Seq.	Mutation	Mut. Freq.
CH12F3-2A+siControl	36/91	63700	91	1.48E-03
L11+sihnRNP L	33/87	60900	89	1.51E-03

C. Analysis of SHM of the V4-39/JH5 region in BL2-JP8Bdel-ER cells treated with indicated siRNAs after AID activation.

Sample	Mut.clone/Total	Total Seq.	Mutation	Mut. Freq.
BL2+siControl	23/61	45750	35	7.65E-04
BL2+sihnRNP K	15/56	42000	19	4.52E-04
BL2+sihnRNP L	30/53	39750	56	1.41E-03

Fig. S3. Summary of SHM analysis of the 5' Su region in CH12F3-2A cells (A and B) and the rearranged V region in BL2-JP8BdelER cells (C). (See Fig. 2.)

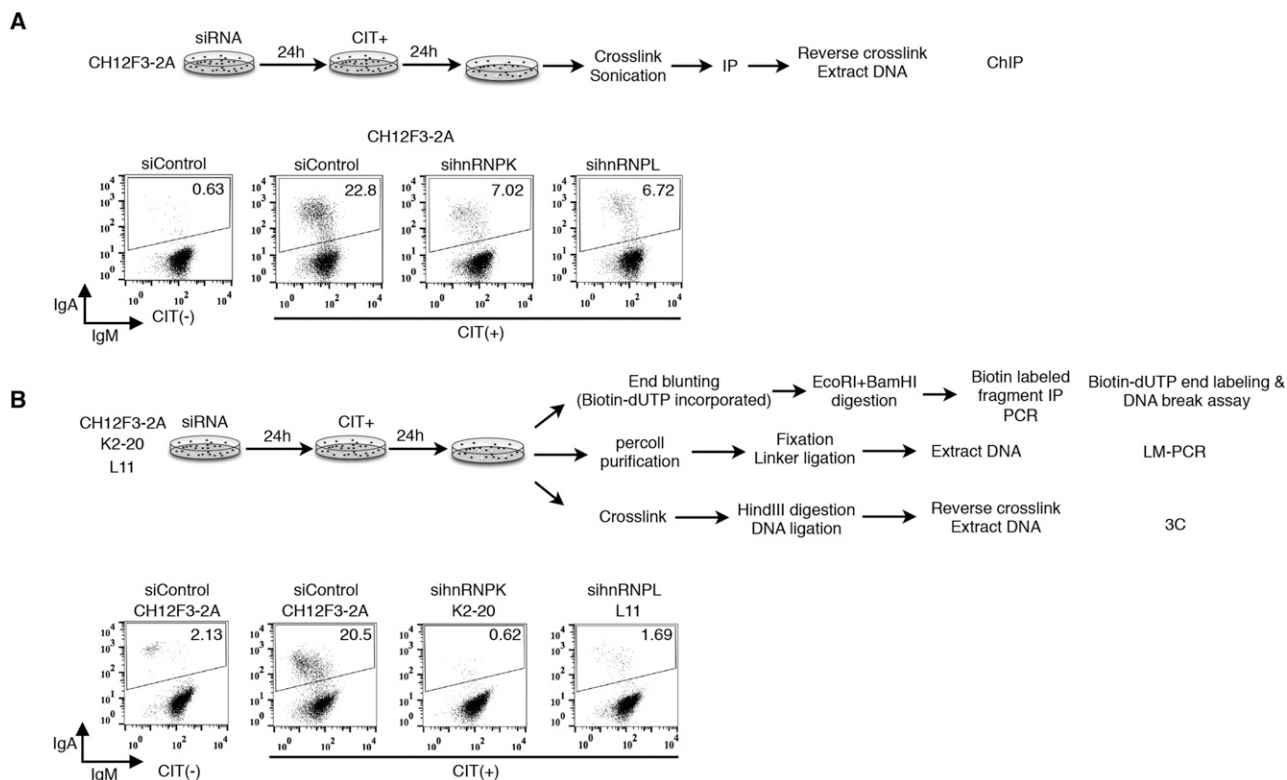


Fig. S4. Experimental designs of the various DNA break assays and the 3C assay. Representative FACS profiles of the cells used in the CSR, DNA break, or 3C assay. (See Fig. 3.)

Purpose	Nucleotide sequence
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Table S1. Cont.

Purpose	Nucleotide sequence
I μ -R	CACAACCATACATTCCCAGGT
S μ -F	CAATGTGGTTTAATGAATTGAAGTTGCCA
S μ -R	TCTCACACTCACCTTGGATCTAAGCACTGT
S α -F	TGAAAAGACTTTGGATGAAATGTGAACCAA
S α -R	GATACTAGGTTGCATGGCTCCATTACACA
Biotin-dUTP end labeling	
S μ -F	AAGCTTGCTGAGCAAAATTAAGGGAACAAGG
S μ -R	GTCTACTTCATTTTGGCTCAAGCCCAGCTTT
S γ 1-F	AGTGTGGGAACCCAGTCAAA
S γ 1-R	GTACTCTCACCGGGATCAGC
DNA break assays (Fig. 3)	
β 2M-F	GGTGACGACCTCCGGATCTG
β 2M-R	GCCGAGTAGCAGCCACTGAAA
LM-PCR	
LMPER.1	GCGGTGACCCGGGAGATCTGAATTC
LMPER.2	GAATTCAGATC
5' S μ -F	GCAGAAAATTAGATAAAATGGATACCTCAGTGG
5' S μ -R	LMPER.1
5' S μ -probe	DIG-AGGGACCCAGGCTAAGAAGGCAAT
GAPDH (forward)	ATCCTGTAGGCCAGGTGATG
GAPDH (reverse)	AGGCTCAAGGGCTTTTAAGG
3C assay	
E μ	GGAACAATTCCACACAAAGACTC
E α	CAAGGTGTTAGGAAAACCTTGCTC
Synapsis by 3C	
S μ	GCTGACATGGATTATGTGAGG
S γ 1 (Fig. 3)	CGACACTGGGCAGTTCATTTTG
S α	GAGCTAGGCTAGACTTACTAAGC
GAPDH3c (forward)	AGTAGTGCGTTCTGTAGATTCC
GAPDH3c (reverse)	CAGTAGACTCCACGACATAC
hnRNP K NM_025279.1, bases 1334–1357	
WT	TCCCAAAGATTGGCTGGATCTAT
Modified	CCCGAAGGACCTAGCGGGCTCCAT
siRNA target site modification hnRNP L NM_177301.5, bases 939–963	
WT	CCTGGGAGATCATCCCGCAGAAATAT
Modified	GCTAGGTGACCACCTGCGGAGTAC

Table S2. siRNA oligos

siRNA oligo	Catalog no.	Nucleotide sequence
Human		
hnRNP K	HSS179311	UCCCAAAGAUUUGGUGGUAUCUAUU
hnRNP L	HSS104917	GGAGCGUGAACAGUGUGCUUCUCUU
Mouse		
hnRNP K	MSS205172	UCCCAAAGAUUUGGUGGUAUCUAUU
hnRNP L	MSS236779	CCUGGGAGAUCAUCCCGCAGAAUUAU
hnRNP M	MSS233822	GGCAGUCACUUAAGACUGGUUAA
HnRNP Q	MSS226121	GCAAGCAGCAAAGAAUCAAUGUAU

Table S3. Antibodies

Antibody	Company	Catalog no.
hnRNP K(D-6)	Santa Cruz	sc-28380
hnRNP L(D-5)	Santa Cruz	sc-48391
hnRNP M1-4(1D8)	Santa Cruz	sc-20002
c-Myc(9E10)	Santa Cruz	sc-40
CD4-FITC(RPA-T4)	eBioscience	11-0049
IgM-FITC	eBioscience	E00715-1631
IgA-PE	Southern Biotech	C2904-W100
FLAG	Sigma	F1804
mAID	eBioscience	K10211
Tubulin	Calbiochem	CP06
phospho-H2A.X(Ser139)	Millipore	05-636

Table S4. Constructs

Construct name	Description
pcDNA3.1-hnRNP K-cMyc-FLAG (wt-K ^R)	WT mouse hnRNP K
pcDNA3.1-ΔKH3 ^R -cMyc-FLAG	KH3 deletion mutants
pCMV6-hnRNP K-cMyc-FLAGg	WT mouse hnRNP K
pCMV6-ΔKH1 ^R -cMyc-FLAG	KH1 deletion mutants
pCMV6-ΔKH2 ^R -cMyc-FLAG	KH2 deletion mutants
pCMV6-ΔKH3 ^R -cMyc-FLAG	KH3 deletion mutants
pCMV6-KH1 ^R -cMyc-FLAG	KH2 and KH3 deletion mutants
pCMV6-KH2 ^R -cMyc-FLAG	KH1 and KH3 deletion mutants
pCMV6-KH3 ^R -cMyc-FLAG	KH1 and KH2 deletion mutants
pCMV6-hnRNP L-cMyc-FLAG (wt-L ^R)	WT mouse hnRNP L
pCMV6-Δ12-L ^R -cMyc-FLAG	Atypical RRM1 and aRRM2 deletion mutants
pCMV6-Δ34-L ^R -cMyc-FLAG	Atypical RRM3 and aRRM4 deletion mutants
pCMV6-Δ234-L ^R -cMyc-FLAG	Atypical RRM2, 3 and 4 deletion mutants
pCMV6-Δ123-L ^R -cMyc-FLAG	Atypical RRM1, 2 and 3 deletion mutants
pCMV6-Δall-L ^R -cMyc-FLAG	All atypical RRMs deletion mutants
pCMV-wtAID-3xFLAG	WT human AID
pCMV-AID-ΔN20-3xFLAG	20-aa N-terminal deletion mutant
pCMV-AID-ΔN50-3xFLAG	50-aa N-terminal deletion mutant
pCMV-P20-3xFLAG	34-aa C-terminal insertion mutant (natural mutant)
pCMV-JP8Bdel-3xFLAG	16-aa C-terminal deletion mutant (natural mutant)